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(Name and address of applicant, and in case of body corporate-place of incorporation)

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Synthetic peptides and pharmaceutical compositions comprising them

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This 31 of August 1998 of the year

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9803

SYNTHETIC PEPTIDES AND PHARMACEUTICAL COMPOSITIONS COMRISING THEM

פפטידים סינתטיים ותכשירי רוקחות המכילים אותם

Yeda Research and Development Co. Ltd.

ידע חברה למחקר ופתוח בע"מ

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to anti-inflammatory peptides derived from pro-inflammatory cytokines, and derivatives thereof, and to pharmaceutical compositions comprising said anti-inflammatory peptides and peptide derivatives.

Migration of inflammatory cells requires cell adhesion and their subsequent detachment from the extracellular matrix (ECM)L. Leukocyte activation and migration must be terminated to stop inflammation. Here we report that IL-2 enhances human T cell adherence to laminin, collagen type IV, and fibronectin (FN). In contrast, neutrophil elastase, an enzyme activated during inflammation, degrades IL-2 to yield IL-2 fractions that inhibit IL-2-induced T cell adhesion to FN. The amino acid composition of two of these IL-2 fractions, which appear to block T cell adherence to FN, were analyzed, and three peptides were consequently synthesized. The three peptides: IVL, RMLT, and EFLNRWIT, but not the corresponding inversely synthesized peptides, inhibited T cell adhesion to FN induced by a variety of activators: IL-2, IL-7, MIP-1β, PMA, as well as anti-CD3 and -β_1 integrin-activating mAb. Moreover, these IL-2 peptides inhibited T cell chemotaxis via FN-coated membranes induced by IL-2 and MIP-1β. Inhibition of T cell adherence and migration apparently involves abrogation of the rearrangement of the T cell actin cytoskeleton. Thus the migrating immune cells, cytokines, and the ECM can create a functional relationship in which both inflammation-inducing signals and inhibitory molecules of immune responses can reciprocally co-exist; the enzymatic products of IL-2 may serve as natural feedback inhibitors of inflammation.

The continuous movement of leukocytes across blood vessel walls to extravascular sites and back to the blood stream is essential for battling foreign pathogens and maintaining homeostasis (1, 2). The migration of T cells through tissues is regulated by adhesion receptors, such as integrins, and by receptors that receive signals provided by pro-inflammatory mediators, such as cytokines, chemokines, and extracellular matrix (ECM[1])-degrading enzymes (3-6). Here we have examined the interactions between human T cells and two molecules, neutrophil elastase and IL-2, which appear to be involved in T cell activation and migration through tissues.

IL-2 is a 15.5 kDa glycoprotein that participates in the development of inflammation and in the regulation of apoptosis (7, 8). In addition to its pro-activatory and proliferative roles, IL-2 also induces neutrophil adhesion to umbilical vein endothelial cells in a CD18-mediated manner (9) as well as chemotactic responses in T cells, both directly and via regulating their expression of CC chemokine receptors (10, 11). The IL-2 receptor consists of three distinct membrane chains: the α, β, and γ chains. The ability of IL-2 to induce T cell activation, differentiation, and proliferation involves the β and γ chains of the IL-2 receptor, which are coupled, through their cytoplasmic domains, to intracellular protein tyrosine kinases and a protein serine/threonine kinase (8, 12). X-ray crystallographic analysis and deletion experiments showed that the sites of IL-2 that bind to the β or γ chains of its receptor are located within the α-helical and 30 amino acid residues of the N-terminal domain of IL-2 (13). Indeed, anti-IL-2 antibodies that recognize amino acid epitopes in the N-terminal region of IL-2 can inhibit IL-2-induced lymphocyte proliferation. The C-terminal portion of IL-2 and its three Cys residues seem to contribute to the folding and active conformation of IL-2 (13, 14).

The majority of neutrophil elastase (also termed human leukocyte elastase; HLE), which exists as either a membrane-bound or soluble moiety, is produced and released by neutrophils, although small amounts are also produced by macrophages, monocytes, and T cells (15, 16).

Elastase degrades basement membrane and ECM glycoproteins, such as elastin, collagen, and fibronectin (FN), as well as molecules expressed on the surface of T cells, e.g., CD4, CD8, and CD2 (17). Recently, two novel functions of neutrophil elastase have been shown: (i) membrane-bound elastase modulates immune cell adhesiveness by interacting with the integrin $\alpha_M\beta_2$ on neutrophils (18), and (ii) elastase processes IL-8, and thus alters the biological functions of this chemokine (19).

Recently, we have shown that IL-7 interacts with ECM, and that both soluble and matrix-complexed IL-7 induce integrin-mediated adhesive interactions of human T cells with ECM-bound IL-7 and with purified VCAM-1 molecules (21). The biological effects of IL-7 are linked to its interaction with the γ-chain of the IL-2 receptor, which is a tyrosine kinase signal-transducing molecule (22). Also, exposure of T cells (3 to 5 days) to IL-2 was found to induce their migration on collagen (23). Therefore, we examined whether IL-2 can induce T cell adhesion to ECM glycoproteins, whether elastase can process recombinant human IL-2, and whether such putative IL-2 peptides can affect T cell-ECM interactions. We found that IL-2 can indeed induce T cell adhesion to ECM glycoproteins that are otherwise non-adhesive moieties. In addition, we may have identified a group of naturally occurring, elastase-generated IL-2 fractions and peptides capable of inhibiting T cell adhesion and migration.

SUMMARY OF THE INVENTION

Migration of immune cells is associated with the secretion of various extracellular matrix (ECM)-degrading enzymes such as heparanase, metalloproteases and elastase, which are secreted by various leukocytes upon activation.

Heparanase cleaves heparan sulfate moieties of the ECM, which facilitates cell migration into tissues (Gilat et al., 1996, Counter-interactions between tissue-infiltrating T lymphocytes, pro-inflammatory mediators, and enzymatically-modified extracellular matrix, Immun. Today, 17:16-20; Lider et al., 1995, A disaccharide that inhibits tumor necrosis factor-α is formed from the extracellular matrix by the enzyme heparanase, Proc.Natl. Acad. Sci.USA, 92: 5037-5041; PCT Publication WO 94/11006 of the applicants).

In addition to the ECM-degrading activities of heparanase, we assumed that heparanase might affect the intensity of inflammation and, in fact, we showed that signaling molecules that inhibit the inflammatory activity of immune cells are produced from the ECM by heparanase (Lider et al., supra; PCT Publication WO 94/11006), indicating that such heparan sulfate-derived oligosaccharides regulate inflammatory reactions by using natural feedback signals that are generated by the immune response itself.

We then proceeded to a working hypothesis that tissue-invading immune cells such as T-cells, can dynamically regulate their functions. Both adhesion- and migration-promoting stimuli, i.e. intact cytokines, and suppressive by-products of these inflammatory mediators may be present, although not necessarily simultaneously, within the inflammatory milieu. At the early stages of inflammation, both pro-inflammatory cytokines and ECM-degrading enzymes may function concomitantly to activate T cells to penetrate tissues. Later on, the peptidic degradation products of the pro-inflammatory cytokines generated by such ECM-degrading enzymes, may inhibit T cell migration, inhibit the co-stimulatory effects of inflammatory cytokine mediators, and therefore signal the termination of the inflammatory reaction. Thus, the source of such a family of anti-inflammatory products of signaling molecules is not limited only to ECM-degraded products as shown in PCT Publication WO 94/11006, but would include cytokine-derived peptidic products.

It has been found according to the present invention that the enzymatic degradation of pro-inflammatory cytokines by proteolytic enzymes that participate in the breakdown of the extracellular matrix (ECM) results in small peptidic fragments that possess anti-inflammatory

activity. This is shown herein in detail for IL-2-derived peptides and preliminary studies carried out with TNF-α show similar results.

The present invention thus relates to anti-inflammatory peptides derived from pro-inflammatory cytokines, and to anti-inflammatory derivatives of said peptides.

The anti-inflammatory peptides derived from pro-inflammatory cytokines of the invention are selected by a method which comprises:

(i) carrying out enzymatic digestion of a pro-inflammatory cytokine with a proteolytic enzyme that participates in the breakdown of the extracellular matrix (ECM);

(ii) testing the fractions obtained in (i) for their in vitro ability to inhibit at least one of the following processes: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; and (d) cytokine secretion by cytokine- or mitogen-stimulated T cells;

(iii) selecting the fractions of (ii) active in at least one of the bioassays (a) to (d), fractionating each fraction further to isolate individual amino acids thereof, and submitting each isolated amino acid to analysis and sequencing;

(iv) synthesizing the amino acids characterized in (iii), carrying out one or more of the bioassays (a) to (d) with each and selecting the amino acids that are active in at least one of said bioassays;

(v) synthesizing the active amino acids selected in (iv) and derivatives thereof;

(vi) submitting the synthetic peptides and peptide derivatives of (v) to in vitro and in vivo assays that measure the anti-inflammatory activity thereof; and

(vii) selecting and producing said peptides and peptide derivatives only if they show anti- inflammatory activity in at least one in vitro and one in vivo assay.

The pro-inflammatory cytokine may be any interleukin, e.g IL-1 to IL-18, any interferon, e.g. IFN-α, IFN-β, IFN-γ, or TNF, and is preferably IL-2 or TNF-α.

The proteolytic enzyme is one capable of processing the glycoprotein constituents of tissues and blood vessel walls and may be elastase, a collagenase or a metalloprotease such as metalloelastase (Chandler et al., 1996, Macrophage metalloelastase degrades matrix and myelin proteins and processes a tumor necrosis factor-α fusion protein, Biochem. Biophys. Res. Commun. 228: 421-429), MMP-2 and MMP-9.

The in vitro bioassays (a) to (d) indicated above are bioassays well-known in the art. Thus, for example, the T cell ahesion assay (a) and the chemotactic migration assay (b) may be carried out as described hereinafter in Materials and Methods; for the T cell proliferation

bioassay (c), purified human T cells ($CD3^+$) are mixed with a mitogen such as PHA (50 ng/ml) or with a cytokine such as IL-2 (100 U/ml), and cultured for 72-96 hours. ^{3}H-thymidine is then added to the culture, and the amount of proliferating cells is determined by gamma counter. The bioassay (d) for inhibition of cytokine secretion, e.g. IFN-γ, TNF-α, IL-2, is carried out in the supernatant of the proliferating T cell cultures by ELISA. Inhibition of the proliferative responses is performed in vitro by adding increased doses of the tested peptides into the proliferative culture mixtures.

In addition to these bioassays, one might use additional bioassays that are specific to a determined cytokine. For example, peptides derived from TNF-α can be tested in an in vitro assay for their ability to inhibit killing of clone 7 fibroblast cells by TNF-α.

The in vivo bioassays to determine anti-inflammatory activity include: (1) in vivo bioassay of inhibition of experimental delayed type hypersensitivity (DTH) reactivity carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.2; (2) treatment of adjuvant arthritis (AA) in rats with the peptides carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.7: and (3) treatment of experimental autoimmune encephalomyelitis (EAE) in guinea pigs, a model disease for multiple sclerosis, reactivity carried out, for example, as described in US Patent No. 5,206,223.

The peptides of the invention include the thus selected peptides derived from a pro-inflammatory cytokine and to peptide derivatives which are obtained by deletion, addition or substitution of one or more amino acid residues, or by substitution of one or more natural amino acid residues by the corresponding D-stereomer or by a non-natural amino acid residue, chemical derivatives of the peptides, cyclic peptides derived from the peptides or from the peptide derivatives, dual peptides and multimers of the peptides.

The peptides and peptide derivatives of the invention are obtained by any method of peptide synthesis known to those skilled in the art, such as for example by solid phase peptide synthesis.

The present invention is also directed to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and at least one anti-inflammatory peptide or peptide derivative of the invention. The pharmaceutical composition will be administered according to known modes of peptide administration, including oral, intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intradermal, transdermal or

other known routes. The dosage administered will be dependent upon the age, sex, health condition and weight of the recipient, and the nature of the effect desired.

The peptides and peptide derivatives of the invention are for use in the treatment of or amelioration of inflammatory disorders including, but not being limited to, autoimmune diseases, e.g. rheumatoid arthritis, diabetes type I, multiple sclerosis, systemic lupus erythematosus, Graves disease; allergy; graft rejection; acute and chronic inflammatory diseases, e.g. uveitis, bowel inflammation.

The present invention further relates to a method of treatment of a patient suffering from an inflammatory disorder which comprises administering to said patient an effective amount of a peptide or peptide derivative of the invention.

Material and methods

Materials. The following reagents were obtained as indicated. Recombinant human IL-2 (sp. act. 18×10^6 U/mg; Chiron B.V., Amsterdam, The Netherlands); recombinant human IL-7 (sp. act. 2×10^5 U/μg; Immunex Corp., Seattle, WA); recombinant human MIP-1β (Pepro Tech, Rocky Hill, NJ); FN (Chemicon; Temecula, CA); BSA, laminin (LN), PMA, and TRITC phalloidin (Sigma Chemical Co., St. Louis, MO); CO-IV (ICN, Costa Mesa, CA); and HEPES buffer, antibiotics, heat-inactivated FCS, sodium pyruvate, and RPMI-1640 (Beit-Haemek, Israel). An anti-β_1 integrin-specific affinity-modulating 8A2 mAb (24), was donated by Dr. J. M. Harlan (Washington University, Seattle, WA). All protected amino acids, coupling reagents, and polymers were obtained from Nova Biochemicals (Läufelfingen, Switzerland). Synthesis-grade solvents were obtained from Labscan (Dublin, Ireland). HPLC solvents and columns were obtained from Merck (Darmstadt, Germany).

T cell adhesion assays. Human T cells were purified from the peripheral blood of healthy donors, and T cell adhesion to immobilized protein substrates was examined as previously described (20, 21). Briefly, human leukocytes were isolated on a Ficoll gradient, washed, and incubated (2 hr, 37°C, 7.5% CO_2, humidified atmosphere) on petri dishes. The non-adherent cells were then collected and incubated (1 hr, 37°C, 7.5% CO_2, humidified atmosphere) on nylon wool columns (Novamed Ltd., Jerusalem, Israel). Unbound cells were eluted from the columns by extensive washings. The resulting cell population was always >92% T cells. Intact IL-2 or IL-2-derived peptides (in 50 μl) were added to flat-bottomed microtiter wells that had been pre-coated with ECM or ECM proteins (FN or LN; 1 μg/well) and blocked with 0.1% BSA. After 4-6 hr at 37°C, the wells were washed and ^{51}[Cr]-labeled T cells were added to the wells, 10^5 cells/100 μl of adhesion medium (RPMI-1640 supplemented with 0.1% BSA, 1% sodium pyruvate, 1% HEPES buffer). The microtiter plates containing the cells were incubated (30 min, 37°C) in a humidified, 7.5% CO_2 atmosphere, and then washed. The adherent cells were lysed, and the resulting supernatants were removed and analyzed in a γ-counter. For each experimental group, the results were expressed as the mean percentage ±SD of bound T cells

from quadruplicate wells. To some wells, different concentrations of soluble IL-2 were added concomitant with the T cells, and with others, different concentrations of elastase-degraded IL-2-derived fractions, or the corresponding synthetic peptides, were added together with stimulators [PMA (50 ng/ml), IL-7 (50 ng/ml), MIP-1β (20 ng/ml), 8A2 (1 μg/ml), mAb anti-CD3 (1 μg/ml), or IL-2 (10 units/ml)].

Chemotaxis assays. T cell chemotaxis was performed and analyzed as previously described (25). Briefly, the migration of human T cells (0.5×10^6 cells in adhesion medium/well) was examined in a 48-well chemotaxis micro-chamber (Neuro-Probe Inc., Cabin John, MD). The two compartments of the micro-chambers were separated by a FN-coated polycarbonate filter (5 μm pore size; OsmonicsProteins Products, Livemore, CA). Where indicated, MIP-1β or IL-2 was added to the lower wells, and the T cells were added to the upper chambers together with the peptides. After incubation (120 min, 37°C, in a humidified, 7.5% CO_2 atmosphere), the filters were removed, fixed, and stained with a Diff-Quik staining kit (Dade, Düdingen, Switzerland). The number of migrating T cells in five high-power fields (under 500x magnification; WILD Microscope, Heerbrugg, Switzerland) was evaluated. For each group the results are expressed as the mean number of cells in one high-power field.

Purification of elastase and elastase digestion of IL-2. Neutrophils were isolated from the whole blood of a healthy donor by dextran sedimentation and Ficoll/hypaque gradient centrifugation, as previously described (26). Elastase was isolated by aprotinin-sepharose affinity chromatography, followed by carboxymethyl-cellulose ion exchange chromatography, as developed by Baugh and Travis (26, 27). The purified elastase, which was lyophilized and stored at -20°C until used, was biochemically checked to be entirely free from cross-contamination with cathepsin G (not shown). IL-2 was dissolved in distilled water to yield a 1 mg/ml solution. Lyophilized neutrophil elastase (50 μg) was dissolved in 1 ml of PBS and immediately added to the IL-2 solution. The elastase-IL-2 mixture was incubated (12 hr) at 37°C. Aliquots were removed and stored at -20°C until subjected to HPLC separation.

Reversed-phase HPLC. Elastase digests of IL-2 were purified with a prepacked Lichrospher-100 RP-18 column (4×25 mm, 5 μm bead size), using a binary gradient formed with 0.1 % TFA in H_2O (solution A) and 0.1% TFA in 75% acetonitrile in H_2O [(solution B) at t=0 min B=3.5%, at t=5 min B=3.5%, and then, the concentrations gegan to increase: at t=60 min B=100% (i.e. 75% acetonitrile)]. The flow-rate was constant on 0.8 ml/min. A Spectra-Physics SP8800 liquid chromatography system equipped with an Applied Biosystems 757 variable wave-length absorbency detector was used. The column effluents were monitored by UV absorbency at 220 nm, and the chromatograms were recorded on a Chrome-Jet integrator. Fractions that were 20% or more above valley levels were pooled, roto-evaporated to a minimal volume, and diluted with HPLC grade water. The roto-evaporation and dilution with water step was performed twice in order to remove residual TFA and acetonitrile.

Amino acid composition of the synthetic peptides and amino acid sequence analysis. Purified peptide solutions [=40 μg of peptide in 40 μl, with 5 μg of norvaline (an unnatural amino acid) as an internal standard] were roto-evaporated, hydrolyzed (10°C, 22 hr) in 6 N HCl under vacuum, and analyzed with an amino acid analyzer (HP1090, Palo-Alto, CA). An on-line pre-column ortho-phthalaldehyde (OPA)/Fmoc derivatisation, combined with reversed phase chromatography, was used to determine the amino acid composition of the peptides and the total peptide yield. Without exception, all the peptides yielded excellent analysis ratios of corresponding amino acids deviation from expected values of less than 10%.

Analysis of the elastase-generated IL-2 fractions was performed using a Model 470A gas-phase microsequencer. Phenylthiohydantoin amino acid derivatives were separated on-line by reversed-phase HPLC on a PTH C-18 column (2.1 x 220 mm) using a Model 120A Analyzer (Applied Biosystems, CA).

Solid phase peptide synthesis. IL-2-derived peptides were prepared by conventional solid phase peptide synthesis, using an ABIMED AMS-422 automated solid phase multiple peptide synthesizer (Langenfeld; Germany). The 9-fluorenylmethoxycarbonyl (Fmoc) strategy was used for peptide chain assembly, according to the commercial protocol. In each reaction vessel

we used 12.5 μmol of Wang resin containing the first, covalently bound corresponding N-Fmoc C-terminal amino acid (typically, polymer loadings of 0.3-0.7 mmols/g resin were used). Fmoc deprotection was achieved by two consecutive treatments with 20% piperidine in dimethyl formamide, usually 10-15 each min at 22°C, depending on the length of peptide and the Fmoc protected amino acid type. The protecting groups used for the side chain of the amino acids were tert-butyloxycarbonyl for Trp, trityl for Asn, and, tert-butyl-ether for Thr. Usually, coupling was achieved using two successive reactions (typically 20-45 min each at 22°C, depending on the length of peptide and amino acid derivative type) with 50 μmol (4 eqv) of N-Fmoc-protected amino acid, 50 μmol (4 eqv) of benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate (PyBop) reagent, and 100 μmol (8 eqv) of N-methylmorpholine were all dissolved in DMF. The peptide was cleaved from the polymer by reacting (2 hr, 22°C) the resin with trifluoroaceticacid/H_2O/triethylsilane; 90/5/5; v/v/v. The solution containing the crude, unprotected peptides was then cooled down to 4°C, precipitated with ether (4°C), and centrifuged (15 min, 3000 rpm, 4°C). The pellet was washed and centrifuged (x3) with ether, dissolved in 30% acetonitrile in H_2O, and lyophilized. The lyophilized material was reconstituted in double distilled water before use; only the stock solution, not the diluted material, was stored at -20°C.

Staining of actin cytoskeleton. T cells were incubated (18 hr, 37°C, 7.5% CO_2, humidified atmosphere) in culture media. IL-2 or PMA was then added and incubated for 24 hr to the cell cultures. The T cells were then washed and seeded onto FN-covered cover slips in the presence of either PMA (50 ng/ml), IL-2 (100 U/ml), or IL-2 peptides (0.1 ng/ml). After 1 hr at 37°C, the adherent cells were fixed (3 min) with paraformaldehyde (3%) and Triton X-100 (0.5%), washed, and fixed (20 min) again with paraformaldehyde (3%). The fixed adherent cells were washed, treated with TRITC phalloidin, and washed again. Photographs (x1000 magnification) were then taken.

Results

Induction of the adhesion of T cells to ECM, FN, LN, and CO-IV by IL-2. Some cytokines induce adhesion of leukocytes to endothelial cells, the underlying basement membrane, and to the ECM (3, 4, 6, 20, 21). Therefore, we examined the ability of soluble IL-2 to induce adhesion of human T cells to ECM, FN, LN, and CO-IV. The results indicated that IL-2 induced T cell adhesion to FN, LN, and CO-IV (Figure 1A), as well as to intact ECM (Figure 1B). Note that the adhesion of T cells to LN induced by IL-2 was lower than that induced to the other ECM glycoproteins. When T cells were activated only with PMA, 45±4.4% of them adhered to immobilized ECM and ECM glycoproteins (not shown). IL-2-induced T cell adhesion to the ECM glycoproteins was inhibited by anti-human β_1 integrins mAb (not shown), which suggests that the pro-adhesive effects of IL-2 were induced via cell surface-expressed integrins. However, under our experimental conditions, IL-2 did not alter the T cell surface expression of β_1 integrins (not shown). Thus IL-2, in addition to other pro-inflammatory mediators, appears to regulate the adhesiveness of resting human T cells to immobilized ECM and ECM glycoproteins.

The effects of IL-2 fragments obtained by elastase degradation on the interaction of T cells with FN. We have assumed that the degradation of IL-2 can occur in the inflammed milieu, where both cytokines, such as IL-2, and proteolytic enzymes, such as neutrophil elastase, are present. We also hypothesized that, in contrast to the intact IL-2 molecule, certain portions of IL-2 can abrogate the adhesiveness of activated T cells to ECM ligands. Hence, elastase and soluble IL-2 were incubated together at physiologic conditions. HPLC analysis of the elastase-degraded IL-2 revealed at least 8 peaks of IL-2, each of which represented at least one low molecular weight protein fragment (Figure 2).

Next, we examined the ability of the HPLC-purified IL-2 fractions, which were generated by elastase-degradation, to inhibit soluble IL-2-induced interactions of T cells with FN. We chose to investigate the major peaks of HPLC-purified, elastase-degraded fractions of IL-2. Fractions 2, 7, and 8 inhibited the adhesion of T cells to immobilized FN in a dose-dependent

and statistically significant fashion, whereas fractions 4 and the HPLC buffer did not (Figure 3). Thus, certain IL-2 fragments, obtained by neutrophil elastase-processing of the cytokine, can inhibit IL-2-induced adhesion of T cells to FN.

The effect of synthetic peptides, with putative amino acid compositions corresponding to fractions 2 and 8, on IL-2-induced T cell adhesion to FN. Next, the primary sequence of fractions 2 and 8 were analyzed by gas-phase chromatography since these elastase-generated fractions of IL-2 appeared to contain adhesion-suppressive peptides. Our analysis revealed that fragment 2 contained an Ile-Val-Leu (IVL; IL-2-112-114) and an Arg-Met-Leu-Thr (RMLT; IL-2-58-61) peptide, whereas fragment 8 contained a Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (EFLNRWIT; IL-2-136-143) octa-peptide. These three peptides were synthesized, and their effects on IL-2-induced T cell adhesion to FN were studied. The IVL, RMLT, and EFLNRWIT, inhibited, in a dose-dependent manner, the adhesion of IL-2-activated T cells to FN; the RMLT is apparantly the most potent inhibitor (Figure 4A). Maximum inhibition was achieved with about 0.1 pg/ml (0.2 pM) for RMLT, and 1 pg/ml of either IVL or EFLNRWIT (2.91 and 0.92 pM, respectively). The inhibitory dose-response curves of IVL and EFLNRWIT are similar to those of the HPLC fractions 2 and 8, respectively, from which they were derived.

To examine the specificity, on the biological and chemical levels, of the inhibition of T cell adhesion by the elastase-generated, synthetic IL-2 peptides, we synthesized the three IL-2 peptides in their inverse amino acid sequences, LVI, TLMR, and TIWRNLFE, and then tested their effects on IL-2-induced T cell adhesion to FN. The results, shown in Figure 4B, indicate that none of these peptides, tested in a broad range of dosages, interfere with T cell adhesion. Thus, the anti-adhesive effects of IVL and EFLNRWIT peptides of IL-2 appear to be due to their direct biological effect on responding lymphocytes.

Do the IL-2 peptides, IVL, RMLT, and EFLNRWIT have to be present during the entire period of the assay to exert their inhibitory effects? The results, shown in Figure 4C, indicate that most of the anti-adhesive effects of the three peptides persevered even if these peptides (at 1

pg/ml) were removed from the T cells prior to their activation with IL-2 and seeding onto the FN-coated surfaces. Apparently, their prolonged inhibitory potential may involve active intracellular signaling pathways. These results suggest that the IL-2 peptides neither exert their inhibitory activities on T cell adhesion to FN via binding to the ECM protein, nor to FN-specific β_1 integrins expressed on the adhering T cells.

IVL, RMLT and EFLNRWIT inhibit T cell adhesion to LN, CO-IV and FN. The next experiment was designed and performed to verify that the three IL-2-derived peptides indeed affect T cell interactions with ECM glycoproteins other than FN. T cells were pre-exposed to the three peptides (at 10 pg/ml), and then activated with IL-2. The treated cells were then added to microtiter wells coated with CO-IV, LN, and FN. The results, shown in Figure 5, indicate that both IVL, RMLT, and EFLNRWIT inhibit T cell adhesion to the three major cell-adhesive glycoproteins of the ECM (Figure 5), suggesting that the elastase-generated IL-2 peptides exert their inhibitory effects over different subsets of β1 integrins.

IVL and EFLNRWIT inhibit T cell chemotactic migration induced by IL-2 or MIP-1β. Immune cell migration is the outcome of a subtle biological equilibrium existing between adhesion and detachment events. Lymphocyte adhesion to the subendothelial ECM and subsequent migration are two active processes that can overlap, but are not mutually dependent events. Adhesion and migration may depend on the ability of the T cells to continuously integrate different pro- and anti-adhesive signals via their versatile receptors for ECM, chemokines, cytokines, and possibly, also antigenic moieties (1, 2). In fact, it has been recently shown that transient (i.e. low affinity) rather than prolonged interactions between integrins and the ECM favor IL-4-induced B cell migration, and not adhesion (28). Therefore, we next examined the effects of the IVL, RMLT, and EFLNRWIT peptides (at 1 pg/ml) on the IL-2- and MIP-1β-induced T cell chemotaxis through FN-coated polycarbonate membranes. The gradient generated by MIP-1β and IL-2, which were placed in the lower compartment of the 48-well chemotaxis apparatus, induced a marked T cell migration through FN-coated membranes, which was about 3 to 4-fold higher than the control (Figure 6). Both IVL, RMLT, and

EFLNRWIT markedly ($P<0.01$) inhibited T cell migration towards IL-2, by about 30, 90, and 60%, respectively. However, although the IVL peptide, and to a lesser degree also the RMLT peptide, markedly inhibited (80% and 60%, respectively) T cell chemotaxis towards MIP-1β, the EFLNRWIT showed only a limited inhibitory effect on the chemokine-induced T cell chemotaxis. Thus, in addition to the capacity of the IVL and EFLNRWIT peptides to inhibit T cell-ECM adhesion, they seem to inhibit T cell migration through FN in response to a diffusible gradient produced by IL-2 or MIP-1β.

Inhibition by the IVL and EFLNRWIT peptides of T cell adhesion to FN induced by various pro-inflammatory mediators and by mAb specific for molecules expressed on T cells. The preceding chemotaxis experiments indicated that the IVL and EFLNRWIT peptides can inhibit T cell adhesion and migration through FN barriers induced not only by IL-2, but also by the chemokine MIP-1β. Therefore, in an attempt to further understand the possible physiological relevance of such phenomena, we examined the ability of these peptides to inhibit the adhesion to FN of T cells stimulated by modes other than IL-2. T cell adhesion to FN can be up-regulated by physiological activators, such as IL-7 (21), MIP-1β (20), and via the CD3 complex, as well as by non-physiological stimuli of integrin avidity, such as PMA (29), and a β_1 integrin-specific activating mAb, 8A2 (24). This mAb up-regulates the endothelial cell and ECM ligand-binding activities of the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins by binding to their cell surface-expressed sites, and thus converting these non-binding integrins to their high affinity, ligand-occupying forms (24).

At 10 pg/ml, both EFLNRWIT and IVL inhibited T cell adhesion to FN that was induced by various stimulators of T cells and modulators of the β_1 integrin functions tested (Figure 7). However, at 0.1 pg/ml, EFLNRWIT did not inhibit the PMA and 8A2-induced adhesion, and the IVL peptide did not inhibit PMA, 8A2, and the anti-CD3 mAb-induced T cell adhesion to FN, which indicates that these modes of activation are less susceptible to IL-2-derived peptide-induced suppression than IL-2-mediated activation. In experiments similar to those shown in Figures A and B, the control peptides (LVI and TIWRNLFE) did not affect T cell adhesion to FN induced by the indicated activators (data not shown). Hence, EFLNRWIT and IVL

apparently inhibit T cell adhesion to the FN component of ECM via a common intracellular event that is linked to the regulation of the avidities and affinities of β_1 integrins, and therefore, to their ligand recognition and binding.

Inhibition of the reorganization of the actin cytoskeleton in FN-attached activated T cells by the EFLNRWIT peptide. The adhesion of immune cells to ECM is dependent on the sequestering of the cytoplasmic domains of integrins in focal adhesion sites, together with actin-containing microfilament bundles (30-32). Therefore, we examined the effect of EFLNRWIT on the morphologies of adherent T cells. The T cells were activated with IL-2 or PMA, treated with the IL-2 peptides, and seeded onto FN-coated cover slips. After incubation and fixation, the actin cytoskeleton of attached T cells was stained with TRITC-conjugated phalloidin. The morphologies of FN-bound IL-2- and PMA-activated T cells (Figure 8, A and B) were markedly different from those of non-activated lymphocytes (Figure 8E); the activated T cells appeared spread, and their actin cytoskeleton performed distinct structures typical of ECM-adherent cells. The EFLNRWIT peptide inhibited the redistribution of the actin skeleton in both the IL-2- (Figure 8C) and PMA-treated (Figure 8D) FN-adherent T cells. Control peptides (LVI, TLMR, and TIWRNLFE) did not inhibit the actin reorganization of the activated T cells (not shown). Hence, the adhesion-inhibiting activity of the IL-2-derived peptide EFLNRWIT, similar to the IVL peptide (data not shown), appears to involve inhibition of the redistribution of the actin cytoskeleton, and therefore, changes in cell shape and spreading.

Discussion

Certain pro-inflammatory mediators can exert their migratory effects in solution or while bound to components of blood vessel walls (3, 6, 33). The binding of these mediators to components of tissue barriers probably ensures the mediators' persistence and effectiveness in the vicinity of the inflammation-mediating immune cells themselves. This transient binding also creates migratory paths, which consist of cytokine gradients that affect the movements of leukocytes through vessel walls and the ECM (3, 4). IL-2 and elastase are perhaps prominent mediators of leukocyte extravasation and migration from the vasculature through the ECM to sites of inflammation. Besides inducing activation and proliferation of T cells, IL-2 can increase the expression of the CC-CKR1 and CC-CKR2 chemokine receptors on $CD45^{RO}$ T lymphocytes (11). This suggests that IL-2 may participate in the recruitment of antigen-primed T cells to sites of immune reactions. We have shown that soluble human IL-2 can induce T cell adhesion to intact ECM and several major ECM-glycoproteins. IL-2 can also associate directly with ECM ligands (dissociation constant in the range of 1 μM), and this association facilitates the adhesion of resting human T cells to lower concentrations of the ECM-complexed cytokine (not shown).

We then hypothesized that, in contrast to the pro-adhesive effects of the intact IL-2 molecule, certain short IL-2-derived peptides, which may occur *in vivo*, can inhibit the interactions of T cells with ECM, and that this interference is independent of the peptides' effects on the activation of T cells by IL-2. We also assumed that such moieties of IL-2 can prevent the arrival of T cells at inflammed sites. Neutrophil elastase was a likely candidate for the physiological production of these inhibitory peptides. Elastase, which is enzymatically versatile (17, 18), can be expressed and secreted by the migrating T cells themselves (16), and can transmit mitogenic stimulations from the environment into the responding T cells (15). In fact, LPS- and fMLP-activated neutrophils express catalytically-active membrane-bound elastases, proteinase 3 and cathepsin G on their cell surfaces, which ensure the presence of these enzymes at the leading edge of the tissue-invading cells (34, 35). Other matrix-degrading

enzymes, such as matrix metalloproteinases, cathepsin B, the urokinase-type plasminogen activator, and plasmin can also be bound on the cell surfaces of fibroblasts and migrating cells (5, 36). An important feature of elastase is its ability to act in both soluble and immobilized forms, since a migrating immune cell that expresses immobilized elastase may encounter matrix-bound IL-2, among other cytokines. We have found that the processing of recombinant IL-2 by elastase results in the production of at least eight different bi-products. Three of these products (present in HPLC fractions 2, 7 and 8) inhibited IL-2-mediated T cell adhesion to FN. Amino acid composition analysis and amino acid sequencing revealed that fraction 2 contained the tri-peptide IVL ($IL\text{-}2_{112\text{-}114}$) and the tetra-peptide RMLT ($IL\text{-}2_{58\text{-}61}$), and fraction 8 the octa-peptide EFLNRWIT ($IL\text{-}2_{136\text{-}143}$). The RMLT peptide appeared to be located within the IL-2-binding site of α chain of the IL-2R, whereas the IVL and EFLNRWIT are located at sites far from the receptor-binding sites of IL-2 (7, 8). These peptides, at a pM range of concentrations (i.e. 0.01 to 1 pg/ml), inhibited the IL-2, as well as MIP-1β-induced chemotaxis of human T cells through FN-coated polycarbonate membranes.

The chemoattractive capacity of IL-2 in T cell migration studies *in vitro* has been shown by using bare polycarbonate filters or CO- or Matrigel-coated membranes as immobilized substrates. T cell migration in these systems was proved to be IL-2R β chain-specific and depended on the activities of the matrix-degrading gelatinases (10, 37, 38). Here, in addition to their anti-migratory effects, both peptides of IL-2 inhibited T cell adhesion to FN induced by various physiological and non-physiological stimuli. Nevertheless, both IVL, RMLT, and EFLNRWIT, at 1 to 100 pg/ml, did not interfere with either PHA- or IL-2-mediated proliferative responses of human T cells, nor did these peptides inhibit the secretion of TNFα and IFNγ from these proliferating cells (not shown). Moreover, the inversely synthesized molecules, LVI, TLMR, and TIWRNLFE, did not inhibit T cell adhesion to FN. Thus, the migration- and adhesion-suppressive capabilities of IVL, RMLT, and EFLNRWIT are specific, and are not due to toxic cell death.

How do the elastase-derived IL-2 peptides exert their inhibitory functions? We have demonstrated that the three peptides do not have to be present during the entire period of the adhesion assay, since their anti-adhesive effect was apparent even after their removal from the assay prior to T cell activation with IL-2. This finding also implies that the IVL, RMLT, and EFLNRWIT peptides do not function by interacting with putative cell-adhesive epitopes present on the tested ECM glycoproteins. The existence and the activity of putative T cell surface-expressed receptors specific to the IL-2 peptides described here require additional study. Current data from our laboratory indicate that the IL-2 peptides, when used in relatively high concentrations (i.e. >1 ng/ml), inhibit a chemokine-induced influx of Ca^{+2} ions into the treated human T cells. The lymphocytes thus treated are then rendered non-responsive to various pharmacological and immunological activators of adhesion (A. Ariel, *et al.*, in preparation). However, it seems to be highly unlikely that two of these peptides (IVL and EFLNRWIT) exert their biological functions by interacting with the IL-2R subunits or by directly binding to β_1-specific integrins. The IL-2 peptides interfered with different modes of T cell activation, leading to their adhesion to the tested matrix proteins. Moreover, both peptides, used in a pM range of concentrations, appear to block T cell adhesion not only to FN, mediated predominantly via the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, but also to LN; T cell adhesion to LN was mediated primarily through the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. Interesting, however, is the fact that the RMLT peptide resides within the IL-2Rα-binding site of IL-2; two residues, Arg-58 and Phe-62, which are present within and adjacent to this peptide, respectively, were shown to be critical for IL-2-IL-2R interactions (13, 39). Therefore, it will also be interesting to examine whether the degradation of IL-2 by elastase produces compounds that can interfere with IL-2 binding to its receptor, and the biological outcome of such molecular interactions.

The ability of EFLNRWIT (as well as the other two IL-2 peptides, not shown) to inhibit the PMA- and IL-2-induced T cell adhesion to FN is probably linked to its ability to block the reorganization of the intracellular actin cytoskeleton. Integrin-cytoskeleton associations can modulate cell adhesion to ECM ligands and cell spreading at areas of cell contact with the

substratum and the micro-clustering and redistribution of β_1 integrins on the cell surface at sites of focal adhesion located at the ends of the actin fibers (29, 31, 40). In fact, actin cytoskeleton reorganization, occurring after activation of leukocytes via a Rho-dependent activation of the ζ isoform of protein kinase C (41, 42), is linked to leukocyte emigration. Similar observations were noted for T cell activation by IL-2 (and PMA) and attachment to FN. Although the intracellular mechanisms of action of the elastase-generated fractions and peptides have not yet been determined, we postulate that these proteins effect the adhesion and migration of T cells within the ECM by active inhibition of intracellular signal transduction pathways linked to cytoskeleton organization, resulting in inhibiting the micro-clustering and association of integrins with cytoskeletal elements.

Our findings imply that the tissue-invading T cells themselves can dynamically regulate their functions. Both adhesion- and migration-promoting stimuli (i.e. intact IL-2) and suppressive bi-products of inflammatory mediators can be present, although not necessarily simultaneously, within the inflammatory milieu (43). At the early stages of inflammation, both IL-2 and elastase may function concomitantly to activate T cells to penetrate tissues. Later, the degradation peptide products of IL-2, generated by elastase, may inhibit T cell migration, inhibit the co-stimulatory effects of IL-2 and other mediators, and probably signal the termination of the inflammatory reaction.

Figure legends

Figure 1. Induction of T cell adhesion to FN, LN, CO-IV (A) and ECM (B) by IL-2. [^{51}Cr]-Labeled human T cells were seeded onto FN, LN, CO-IV, and ECM-coated microtiter wells together with IL-2. After 30 min at 37°C, non-adherent T cells were removed, the adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

Figure 2. Chromatogram of IL-2 fractions obtained upon elastase proteolysis. IL-2 (1 mg/ml) was incubated (12 hr, 37°C) with neutrophil elastase (50 µg/ml in PBS). The resulting enzymatic digests were purified by HPLC. Fraction 1 consisted of salts used for the separation procedure. One experiment representative of three.

Figure 3. Effects of IL-2 fractions, generated by elastase degradation, on the IL-2-induced adhesion of T cells to FN. IL-2 (10 U/ml)-stimulated [^{51}Cr]-labeled T cells were seeded, in the presence or absence of elastase-generated IL-2 protein products, onto wells coated with FN. After 30 min at 37°C, non-adherent T cells were removed, and the percentage of adhered cells was determined. One experiment representative of six.

Figure 4. Specific inhibition, by elastase-generated fractions 2 and 8 of IL-2 and by their synthetic peptides, of IL-2-induced T cell adhesion to FN. A. T cell adhesion to FN in the presence of IL-2 fractions and peptides. B. The effects on T cell adhesion to FN of the inversely synthesized IL-2 peptides. T cells were activated with IL-2 (10 U/ml) and seeded onto FN-coated wells in the presence of IL-2, fraction 2, fraction 8, or IL-2 peptides. C. Effects of pre-treatments of T cells with IL-2 peptides on the subsequent IL-2-induced T cell adhesion to FN. T cells used were untreated or pre-treated with the indicated peptides (1 pg/ml; 60 min, 37°C, 10% CO_2, humidified atmosphere), washed (x2), exposed to IL-2, and seeded onto the FN coated wells. After 30 min at 37°C, T cell adhesion was measured. One experiment representative of five.

Figure 5. Inhibition by the IL-2 peptides of T cell adhesion to LN, CO-IV and FN. T cells were pre-treated with the indicated IL-2 peptides (10 pg/ml, 30 min, 37°C, 10% CO_2, humidified atmosphere) and then with IL-2 (10 U/ml). The T cells were then seeded onto microtiter wells which were pre-coated (1 μg/well) with the various ECM glycoproteins. T cell adhesion was measured 30 min later.

Figure 6. Inhibition of IL-2-induced T cell migration through FN by EFLNRWIT and IVL. T cells were pre-treated with IL-2 peptides (1 pg/ml) or buffer alone and then placed in the upper wells of a chemotaxis chamber, in which IL-2 (10 U/ml) or MIP-1β (10 ng/ml) had been added to the lower compartment. T cell migration towards the chemotactic sources was assessed after 2 hr. One experiment representative of four.

Figure 7. Inhibition by EFLNRWIT (A) and IVL (B) of T cell adhesion to FN, induced by various activators. Labeled T cells were seeded onto FN-coated wells, in the presence of IL-2 (10 U/ml), IL-7 (50 ng/ml), MIP-1β (20 ng/ml), PMA (50 ng/ml), 8A2 mAb (1 μg/ml), or anti-human CD3 mAb (1 μg/ml). The IL-2-derived peptides, IVL or EFLNRWIT were also present in some wells. After 30 min at 37°C, non-adherent T cells were removed by washing, the remaining adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

Figure 8. Inhibition by the EFLNRWIT peptide of the spreading and redistribution of the actin cytoskeleton in IL-2- and PMA-activated, FN-adherent T cells. T cells were activated (48 hr) with IL-2 (50 U/ml). The T cells were then washed and seeded onto FN-coated cover slips, in medium alone (E), in the presence of IL-2 (100 U/ml; A and C), or PMA (50 ng/ml; B and D), or EFLNRWIT (10 pg/ml; C and D). After incubation, the intracellualr actin filaments of the fixed FN-attached T cells were stained. Original magnification ×1000.

References

1. Butcher, E. C. and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science 272:60.*
2. Clark, E. A. and J. S. Brugge. 1995. Integrins and signal transduction: the road taken. *Science 268:233.*
3. Ben-Baruch, A., D. F. Michiel, and J. J. Oppenheim. 1995. Signals and receptors involved in recruitment of inflammatory cells. *J. Biol. Chem. 270:11703.*
4. Nathan, C. and M. Sporn. 1991. Cytokines in context. *J. Cell Biol. 113:981.*
5. Blasi, F. 1997. uPA, uPAR, PAI-1: key intersection of proteolytic, adhesive and chemotactic highways? *Immunol. Today 9:415.*
6. Gilat, D., L. Cahalon, R. Hershkoviz, and O. Lider. 1996. Counter-interactions between tissue-infiltrating T lymphocytes, pro-inflammatory mediators, and enzymatically-modified extracellular matrix. *Immunol. Today 17:16.*
7. Smith, K. A. 1988. Interleukin-2: inception, impact, and implications. *Science 240:875.*
8. Taniguchi, T. and Y. Minami. 1993. The IL-2/IL-2 receptor system: a current overview. *Cell 73:5.*
9. Li, J., S. Gyorffy, S. Lee, and C. S. Kwok. 1996. Effect of recombinant human interleukin-2 on neutrophil adherence to endothelial cells in vitro. *Inflammation 20:361.*
10. Wilkinson, P. C. and I. Newman. 1994. Chemoattractant activity of IL-2 for human lymphocytes: a requirement for the IL-2 receptor β-chain. *Immunol. 82:134.*
11. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med. 184:569.*
12. Minami, Y. and T. Taniguchi. 1995. IL-2 signaling: recruitment and activation of multiple protein tyrosine kinases by the components of the IL-2 receptor. *Curr. Opin. Cell Biol. 7:156.*

13. Ju, G., I. Collins, K. L. Kaffka, W. H. Tsien, R. Chizzonite, R. Crowl, R. Bhatt, and P. L. Kilian. 1987. Structure-function analysis of human interleukin-2. Identification of amino acid residues required for biological activity. *J. Biol. Chem. 262:5723.*

14. Kuo, L. M. and R. J. Robb. 1986. Structure-function relationships for the IL 2-receptor system. II. Localization of an IL-2 binding site on high and low affinity receptors. *J. Immunol. 137:1538.*

15. Packard, B. Z., H. S. Mostowski, and A. Komoriya. 1995. Mitogenic stimulation of human lymphocytes mediated by a cell surface elastase. *Biochem. Biophys. Acta 1269:51.*

16. Bristow, C. L., L. K. Lyford, D. P. Stevens, and P. M. Flood. 1991. Elastase is a constituent product of T cells. *Biochem. Biophys. Res. Comm. 181:232.*

17. Döring, G., F. Frank, C. Boudir, S. Herbert, B. Fleischer, and G. Bellon. 1995. Cleavage of lymphocyte surface antigens CD2, CD4, and CD8 by polymorphonuclear leukocyte elastase and cathepsin G in patients with cystic fibrosis. *J. Immunol. 154:4842.*

18. Tian-Quan, C. and S. D. Wright. 1996. Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, $\alpha_M\beta_2$) and modulates polymorphonuclear leukocyte adhesion. *J. Exp. Med. 84:1213.*

19. Padrines, M., M. Wolf, A. Walz, and M. Baggiolini. 1994. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett. 352:231.*

20. Gilat, D., R. Hershkoviz, Y. A. Mekori, I. Vlodavsky, and O. Lider. 1994. Regulation of adhesion of $CD4^+$ T lymphocytes to intact or heparinase-treated subendothelial extracellular matrix by diffusible or anchored MIP-1β and RANTES. *J. Immunol. 153:4899.*

21. Ariel, A., R. Hershkoviz, L. Cahalon, C. Chen, D. E. Williams, S. K. Akiyama, K. M. Yamada, R. Alon, T. Lapidot, and O. Lider. 1997. Induction of T cell adhesion to extracellular matrix and endothelial cell ligands by soluble or matrix-bound IL-7. *Eur. J. Immunol. 27:2562.*

22. Kishimoto, K., T. Taga, and S. Akira. 1994. Cytokine signal transduction. *Cell 76: 253*.

23. Ratner, S., P. Patrick, and G. Bora. 1992. Lymphocyte development of adherence and motility in extracellular matrix during IL-2 stimulation. *J. Immunol. 149:681*.

24. Kovach, N. L., T. M. Carlos, E. Yee, and J. M. Harlan. 1992. A monoclonal antibody to β_1 integrin (CD29) stimulates VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. *J. Cell Biol. 116:499*.

25. Ben-Baruch, A., M. Grimm, K. Bengali, G. A. Evans, O. Chertov, J. M. Wang, O. M. Howard, N. Mukaid, K. Matsushima, and J. J. Oppenheim. 1997. The differential ability of IL-8 and neutrophil-activating peptide-2 to induce attenuation of chemotaxis is mediated by their divergent capabilities to phosphorylate CXCR2 (IL-8 receptor B). *J. Immunol. 158:5927*.

26. Yavin, E. J., L. Yan, D. M. Desiderio, and M. Fridkin. 1996. Synthetic peptides derived from the sequence of human C reactive protein inhibit the enzymatic activity of human leukocyte elastase and cathepsin G. *Int. J. Pep. Prot. Res. 48:465*.

27. Baugh, J. and Travis, J. 1976. Human leukocyte granule elastase: rapid isolation and characterization. *Biochemistry 15:836*.

28. Elenstrom-Magnusson, C., W. Chen, B. Clinchy, B. Obrink, and E. Severison. 1995. IL-4-induced B cell migration involves transient interactions between β1 integrins and extracellular matrix components. *Int. Immunol. 7:567*.

29. Shimizu, Y. and S. W. Hunt III. 1996. Regulating integrin-mediated adhesion: one more function for PI 3-kinase? *Immunol. Today 17:565*.

30. Sánchez-Mateos, P., C. Cabañas, and F. Sánchez-Madrid. 1996. Regulation of integrin function. *Sem. Cancer Biol. 7:99*.

31. Newham, P., and M. J. Humphries. 1996. Integrin adhesion receptors: structure, function and implication for biomedicine. *Mol. Med. Today 2:304*.

32. Miyamoto, S., H. Teramoto, O. A. Coso, J. S. Gutkind, P. D. Burbelo, S. K. Akiyama, and K. M. Yamada. 1995. Integrin function: Molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol. 131:791.*

33. Tanaka, Y., D. Adams, and S. Shaw. 1993. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol. Today 14:111.*

34. Owen, C. A., M. A. Campbell, P. L. Sannes, S. S. Boukedes, and E. J. Campbell. 1995. Cell surface-bound elastase and cathepsin F on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J. Cell Biol. 131:775.*

35. Csernok, E., M. Ernst, W. Schmitt, D. F. Bainton, and W. L. Gross. 1994. Activated neutrophils express proteinase 3 on their plasma membrane in vitro and in vivo. *Clin. Exp. Immunol. 95:244.*

36. Plow, E. F., D. E. Freaney, J. Plescia, and L. A. Miles. 1986. The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. *J. Cell Biol. 103:2411.*

37. Leppert, D., E. Waubant, R. Galardy, N. W. Bunnett, and S. L. Hauser. 1995. T cell gelatinases mediate basement membrane transemigration *in vitro. J. Immunol. 154:4379.*

38. Pleass, R., and R. Camp. 1994. Cytokines induce lymphocyte migration *in vitro* by direct, receptor-specific mechanisms. *Eur. J. Immunol. 24:273.*

39. Sauve, K., M. Nachman, C. Spence, P. Bailon, E. Campbell, W. H. Tsien, J. A. Kondas, J. Hakimi, and G. Ju. 1991. Localization in human interleukin 2 of the binding site to the α-chain (p55) of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA . 88:4636.*

40. Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between α-actinin and the β1 integrin subunit in vitro. *J. Cell. Biol. 111:721.*

41. Downey, G. P., C. K. Chan, P. Lea, A. Taiki, and S. Grinstein. 1992. Phorbol ester-induced actin assembly in neutrophils: role of the protein kinase C. *J. Cell Biol. 116:695.*

42. Gomez, J., A. Garcia, L. R. Borlado, P. Bonay, C. Martinez, A. Silva, M. Fresno, A. C. Carrera, C. Eicher-Streiber, and A. Rebollo. 1997. IL-2 signaling controls actin organization through Rho-like protein family, phospotidylinositol 3-kinase, and protein kinase C-ζ. *J. Immunol. 158:1516.*

43. Hauzenberger, D., J. Klominek, S. E. Bergstrom, and K. G. Sundqvist. 1995. T lymphocyte migration: the influece of interactions via adhesion molecules, the T cell receptor, and cyokines. *Crit. Rev. Immunol. 15:285.*

CLAIMS

1. A synthetic anti-inflammatory peptide derived from a pro-inflammatory cytokine, and anti-inflammatory derivatives of said peptide.

2. A synthetic anti-inflammatory peptide according to claim 1, wherein the pro-inflammatory cytokine is selected from interleukins, interferons, and TNFs.

3. A synthetic anti-inflammatory peptide according to claim 2, derived from IL-2.

4. A synthetic anti-inflammatory peptide according to claim 2, derived from TNF-α.

5. A synthetic anti-inflammatory peptide or peptide derivative according to any one of claims 1 to 4, which inhibits at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; and is active in at least one of the following bioassays in vivo: (1) inhibition of experimental delayed type hypersensitivity (DTH) reactivity; (2) treatment of adjuvant arthritis (AA) in rats: and (3) treatment of experimental autoimmune encephalomyelitis (EAE) in guinea pigs.

6. A pharmaceutical composition comprising at least one synthetic anti-inflammatory peptide or peptide derivative according to any one of claims 1 to 5, and a pharmaceutically acceptable carrier.

7. A method of selecting anti-inflammatory peptides derived from pro-inflammatory cytokines, which comprises:

(i) carrying out enzymatic digestion of a pro-inflammatory cytokine with a proteolytic enzyme that participates in the breakdown of the extracellular matrix (ECM);

(ii) testing the fractions obtained in (i) for their in vitro ability to inhibit at least one of the following processes: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; and (d) cytokine secretion by cytokine- or mitogen-stimulated T cells;

(iii) selecting the fractions of (ii) active in at least one of the bioassays (a) to (d), fractionating each fraction further to isolate individual amino acids thereof, and submitting each isolated amino acid to analysis and sequencing;

(iv) synthesizing the amino acids characterized in (iii), carrying out one or more of the bioassays (a) to (d) with each and selecting the amino acids that are active in at least one of said bioassays;

(v) synthesizing the active amino acids selected in (iv) and derivatives thereof;

(vi) submitting the synthetic peptides and peptide derivatives of (v) to in vitro and in vivo assays that measure the anti-inflammatory activity thereof; and

(vii) selecting and producing said peptides and peptide derivatives only if they show anti- inflammatory activity in at least one in vitro and one in vivo assay.

8. The method according to claim 7 wherein the pro-inflammatory cytokine is selected from interleukins, interferons, and TNFs.

9. The method according to claim 8 wherein the cytokine is IL-2 or TNF-α.

10. The method according to any one of claims 7-9 wherein the proteolytic enzyme is elastase, a collagenase or a metalloprotease.

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Patent Attorney

Figure 1 (A, B)

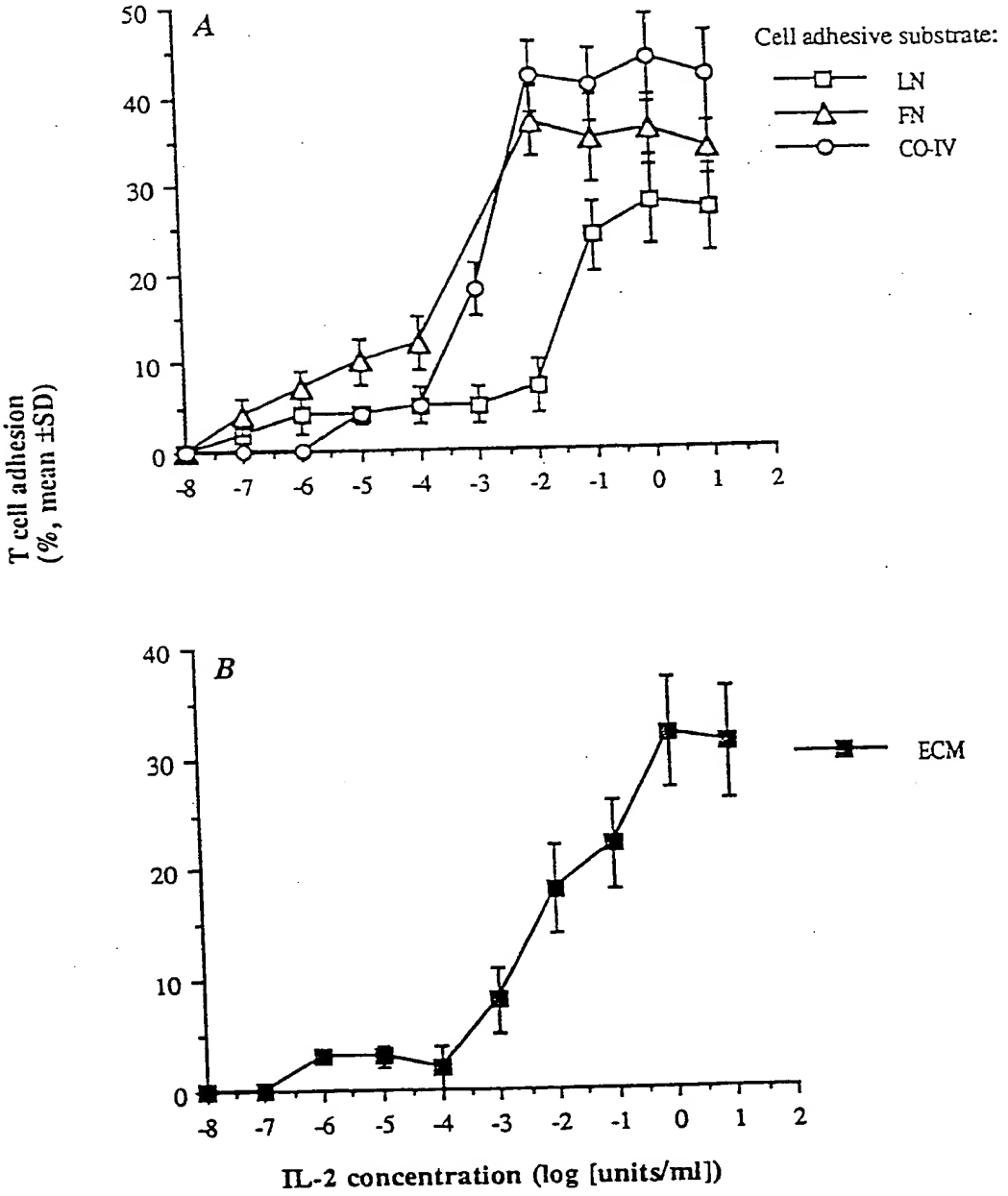
A
B
Cell adhesive substrate:
LN
FN
CO-IV
ECM
T cell adhesion
(%, mean ±SD)
IL-2 concentration (log [units/ml])
50
40
30
20
10
0
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2

Figure 2

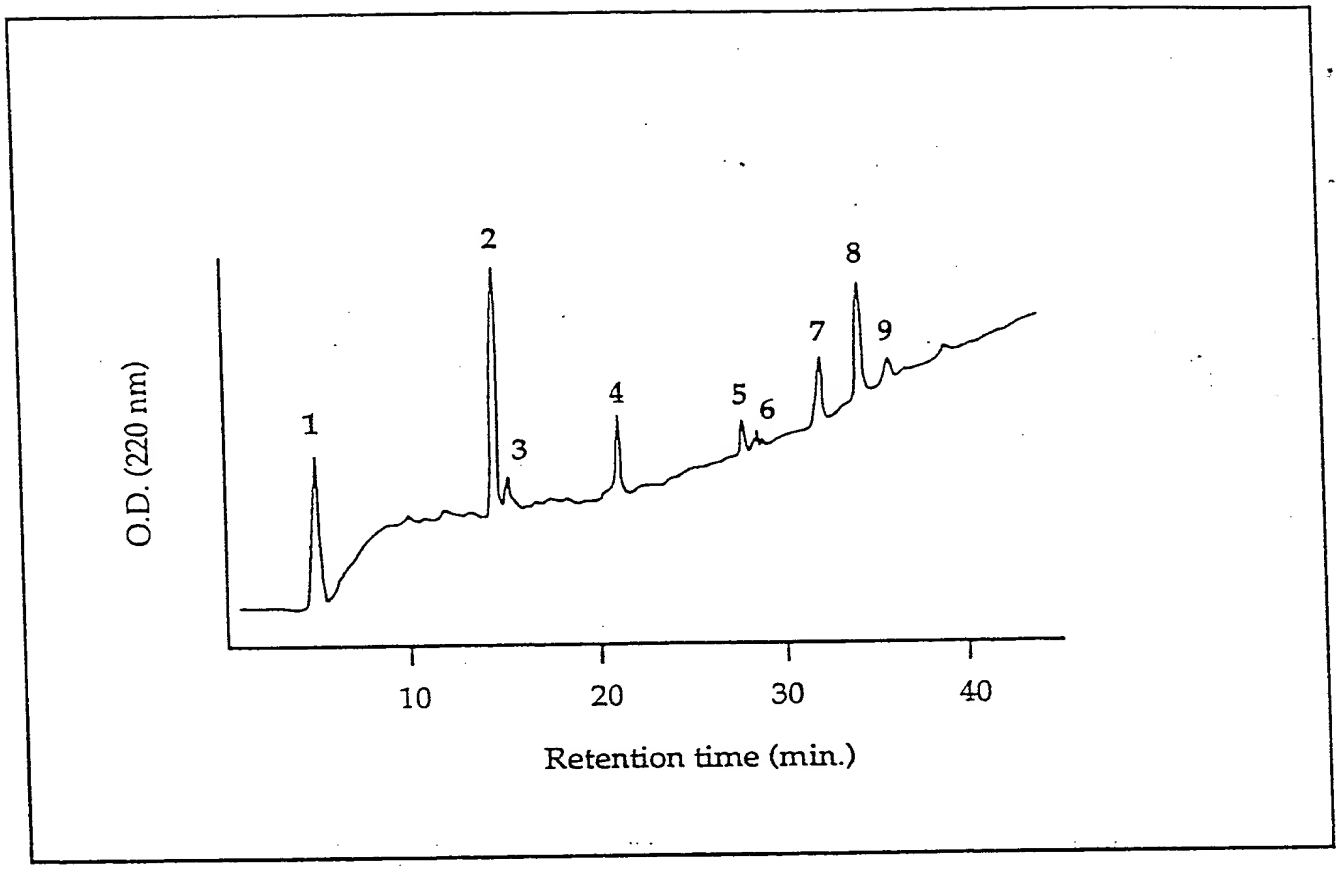

Figure 3

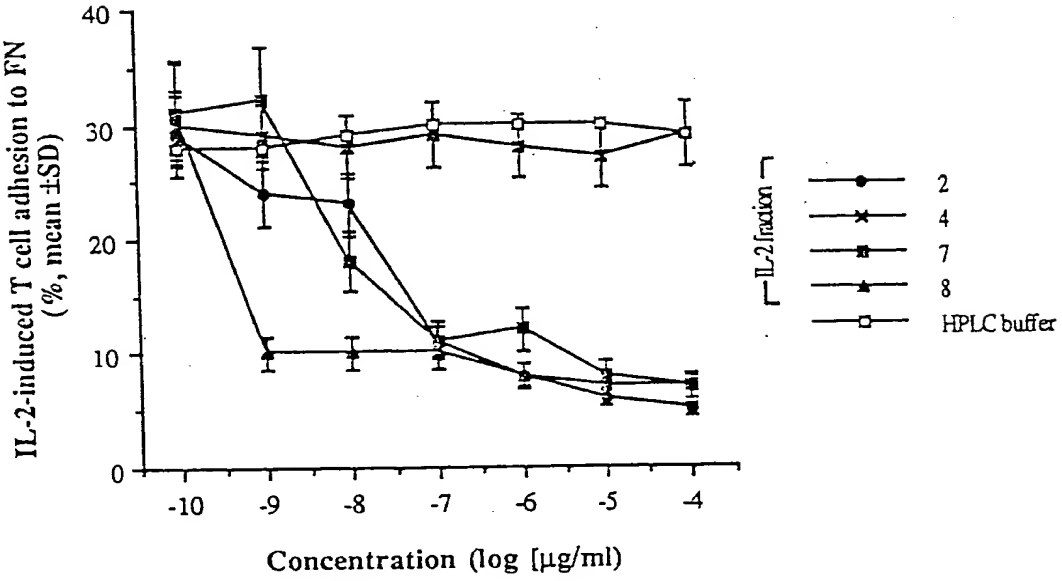
IL-2-induced T cell adhesion to FN
(%, mean ±SD)
40
30
20
10
0
-10
-9
-8
-7
-6
-5
-4
Concentration (log [µg/ml)
IL-2 fraction
2
4
7
8
HPLC buffer

Figure 4

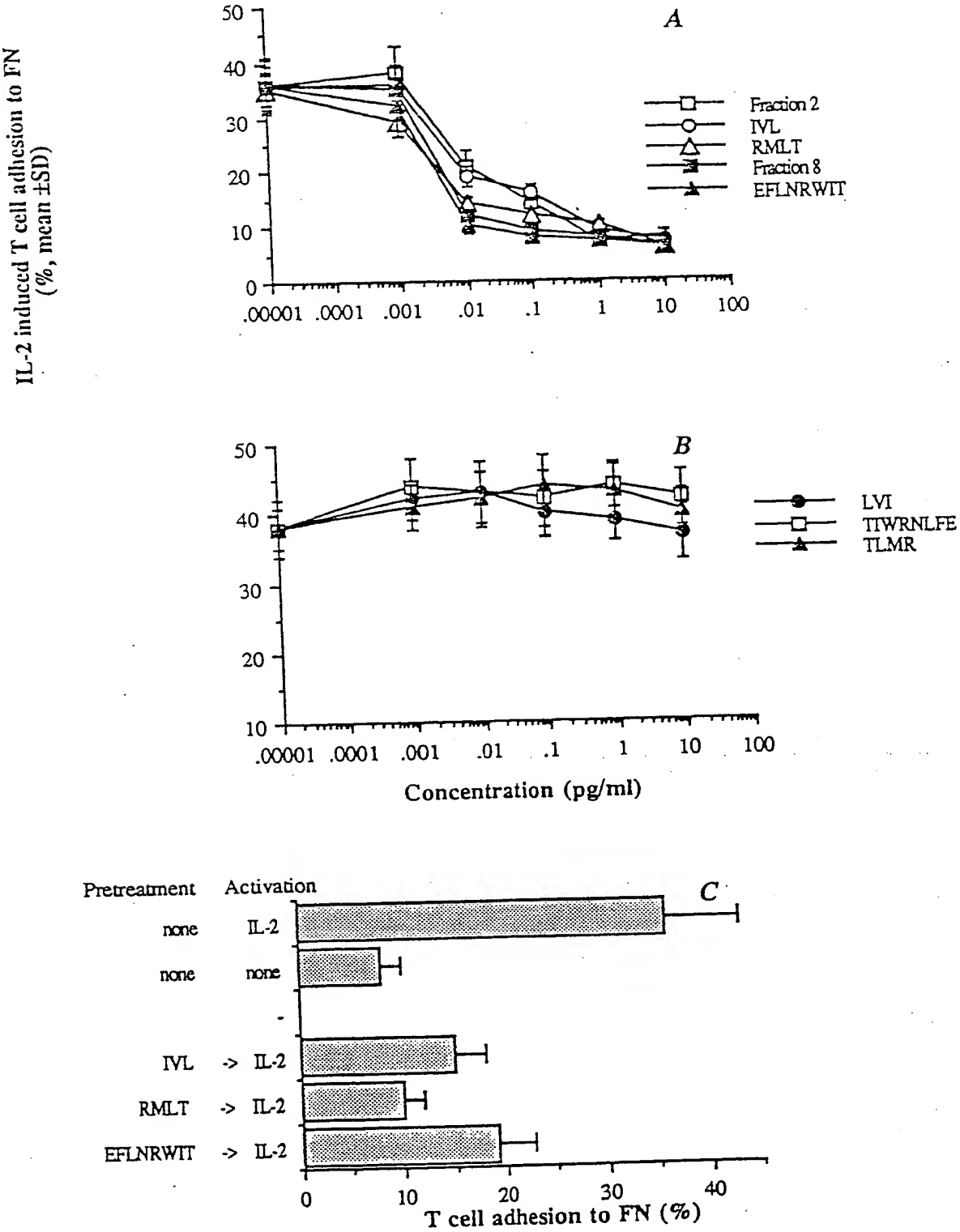
IL-2 induced T cell adhesion to FN
(%, mean ±SD)
A
50
40
30
20
10
0
.00001 .0001 .001 .01 .1 1 10 100
Fraction 2
IVL
RMLT
Fraction 8
EFLNRWIT
B
50
40
30
20
10
.00001 .0001 .001 .01 .1 1 10 100
LVI
TTWRNLFE
TLMR
Concentration (pg/ml)
Pretreatment
Activation
C
none
IL-2
none
none
IVL
-> IL-2
RMLT
-> IL-2
EFLNRWIT
-> IL-2
0 10 20 30 40
T cell adhesion to FN (%)

Figure 5

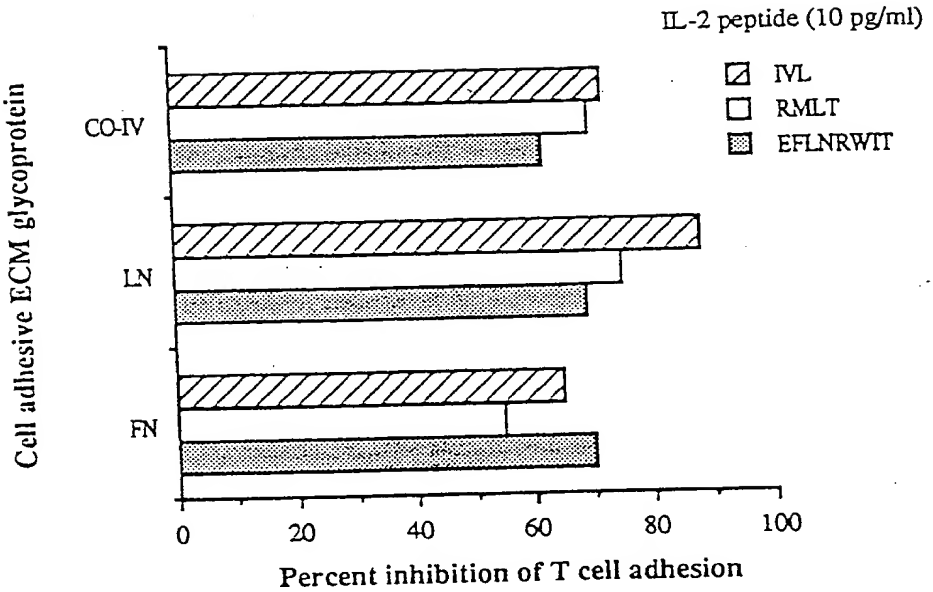
IL-2 peptide (10 pg/ml)
IVL
RMLT
EFLNRWIT
Cell adhesive ECM glycoprotein
CO-IV
LN
FN
0
20
40
60
80
100
Percent inhibition of T cell adhesion

Figure 6

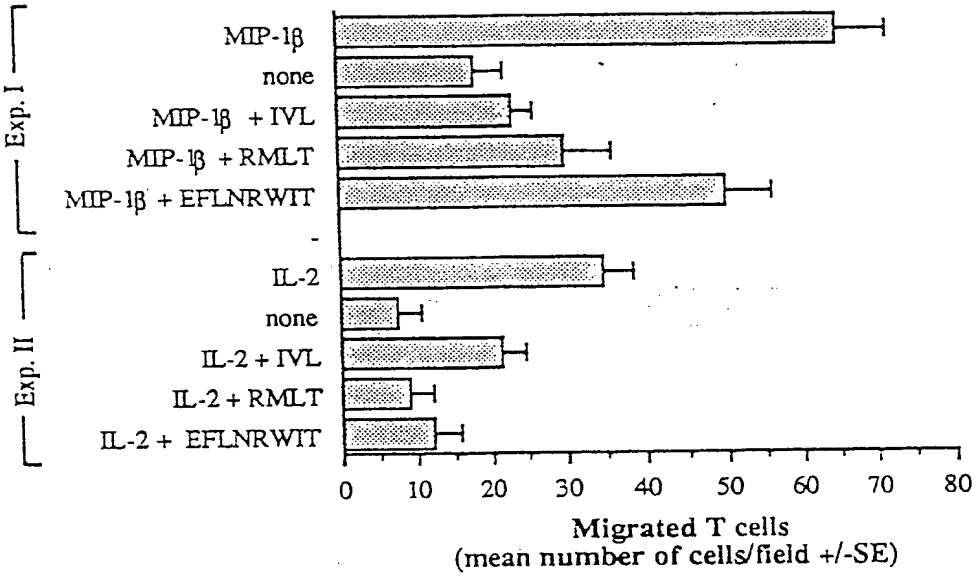
Exp. I
Exp. II
MIP-1β
none
MIP-1β + IVL
MIP-1β + RMLT
MIP-1β + EFLNRWIT
IL-2
none
IL-2 + IVL
IL-2 + RMLT
IL-2 + EFLNRWIT
0
10
20
30
40
50
60
70
80
Migrated T cells
(mean number of cells/field +/-SE)

Figure 7

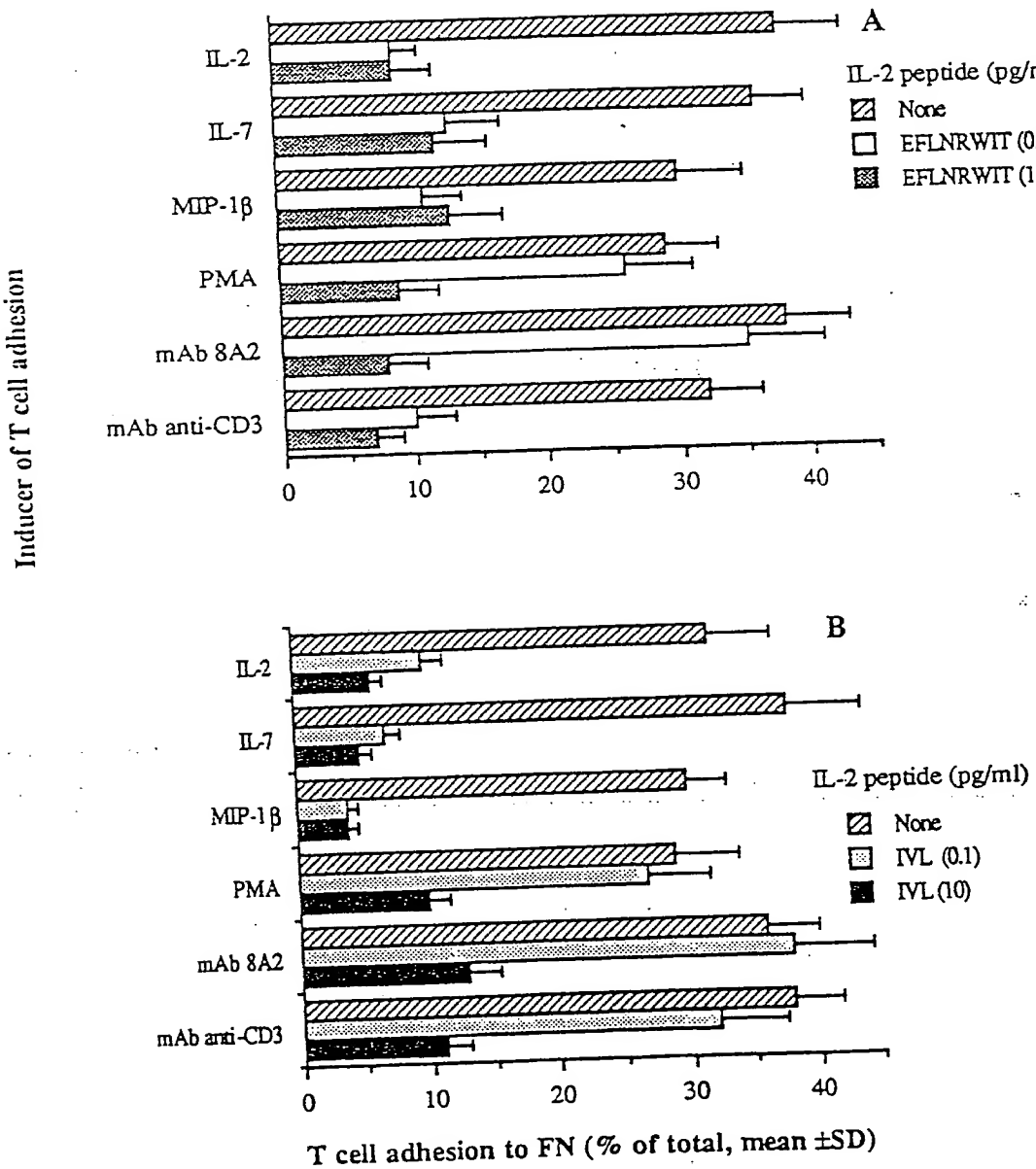
A
IL-2
IL-7
MIP-1β
PMA
mAb 8A2
mAb anti-CD3
0
10
20
30
40
IL-2 peptide (pg/ml)
None
EFLNRWIT (0.1)
EFLNRWIT (10)
B
IL-2
IL-7
MIP-1β
PMA
mAb 8A2
mAb anti-CD3
0
10
20
30
40
IL-2 peptide (pg/ml)
None
IVL (0.1)
IVL (10)
Inducer of T cell adhesion
T cell adhesion to FN (% of total, mean ±SD)

Figure 8